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QIIME2

Anticipated workshop duration when delivered to a group of participants is **4 hours**.

For queries relating to this workshop, contact Melbourne Bioinformatics (bioinformatics-training@unimelb.edu.au).

Overview

Topic

- [x] Genomics
- [ ] Transcriptomics
- [ ] Proteomics

- [ ] Metabolomics
- [ ] Statistics and visualisation
- [ ] Structural Modelling
- [ ] Basic skills

Skill level

- [ ] Beginner
- [x] Intermediate
- [ ] Advanced

This workshop is designed for participants with command-line knowledge. You will need to be able to `ssh` into a remote machine, navigate the directory structure and `scp` files from a remote computer to your local computer.

Description

What is the influence of genotype (intrinsic) and environment (extrinsic) on anemone-associated bacterial communities?

Data: Illumina MiSeq v3 paired-end (2 × 300 bp) reads (FASTQ).

Tools: QIIME 2

Pipeline:

Section 1: Importing, cleaning and quality control of the data
Section 2: Taxonomic Analysis
Section 3: Building a phylogenetic tree
Section 4: Basic visualisations and statistics
Section 5: Exporting data for further analysis in R
Section 6: Extra Information

Learning Objectives

At the end of this introductory workshop, you will:

- Take raw data from a sequencing facility and end with publication quality graphics and statistics
- Answer the question *What is the influence of genotype (intrinsic) and environment (extrinsic) on anemone-associated bacterial communities?*

Tutorial layout

- There is a `Table of contents` on the right-hand side which can be used to easily navigate through the tutorial by clicking the relevant section.

```
These grey coloured boxes are code blocks. The rectangular boxes in the top
right hand corner of this code block/grey box can be used to copy the code to
the clipboard.
```

??? example "Coloured boxes like these with > on the far right hand side, can be clicked to reveal the contents."
REVEALED!

!!! attention "Attention: Pay attention to the information in these boxes."
Important information, hints and tips.

Requirements and preparation

!!! attention "Important"
Attendees are required to use their own laptop computers.

```
At least one week before the workshop, if required, participants should install the software below.  This sho
```

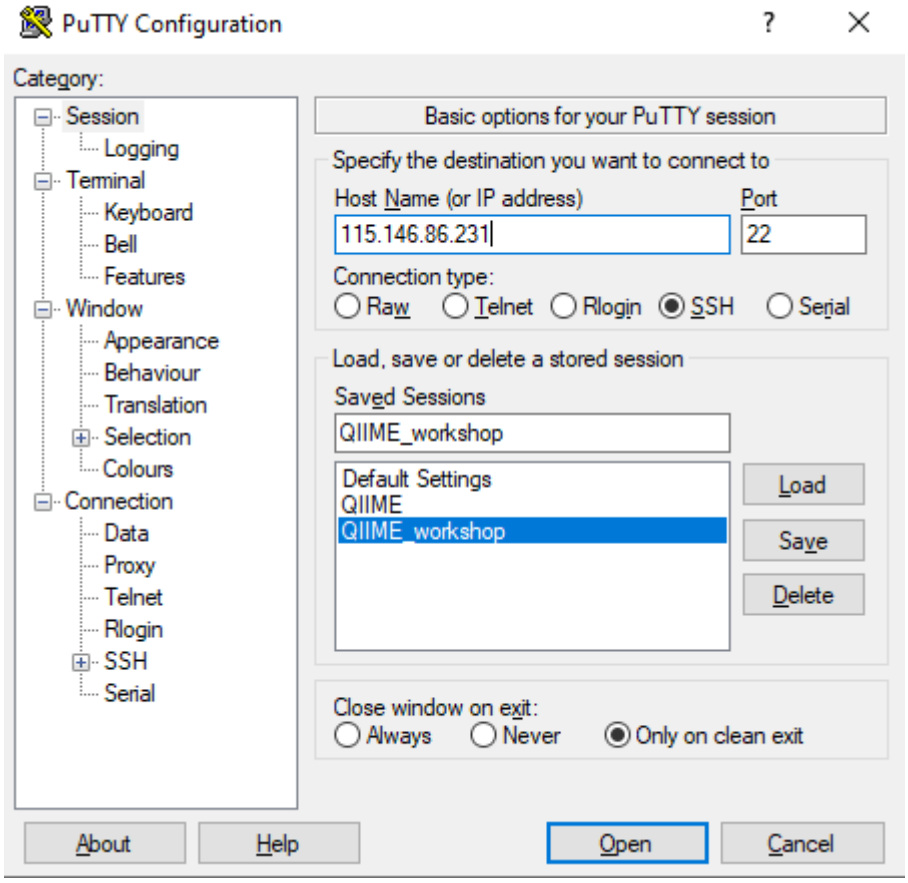
Required Software

Mac Users: No additional software needs to be installed for this workshop. Software for file transfers between a local computer and remote server such as WinSCP or FileZilla can be used.

Windows Users:

1. A terminal emulator such as PuTTY (free and open-source) will need to be downloaded.

??? example "Putty Example"



2. Software for file transfers between a local computer and remote server such as WinSCP or FileZilla.

Mode of Delivery

This workshop will be run on a Nectar Instance. An “Instance” is Nectar terminology for a virtual machine running on the Nectar Cloud OpenStack infrastructure. An “Instance” runs on a “compute node”; i.e. a physical computer populated with processor chips, memory chips and so on.

You will be given an individual username, IP address and password to log on to using the SSH client tool on your computer (Terminal on Mac or PuTTY on Windows).

```
ssh username@nectar_ip-address
```

Should you wish to do this tutorial at a later stage independently, it is possible to apply for your own instance directly through a Nectar allocation. There are also many helpful Nectar Research Cloud tutorials.

Byobu-screen

Some of the commands in this tutorial take a while to run. Should your connection drop and the SSH session on Nectar terminates, any commands that are running will terminate too. To mitigate this, once logged on to the Nectar Instance, we'll run `byobu-screen` (an enhancement for the `screen` terminal multiplexer) which allows us to resume a session. In other words, processes running in `byobu-screen` will continue to run when their window is not visible, even if you get disconnected.

On Nectar, to start a `byobu-screen` session called `workshop`, type

```
byobu-screen -S workshop
```

??? example "Byobu Example"

```
(qiime2-2021.4) epsilon@test-i5:~$
```

```
0*$ bash                                                    epsilon@test-i5 115.146.84.199 Menu:<F9>
u  Ubuntu 20.04                    (R) 9! 1## 2d4h 0.00 16x2.0GHz 31.4GB3% 30GB27% 2021-08-18 04:50:22
```

You can then proceed to run the commands in the workshop as normal.

Should your SSH session on Nectar terminate, once you log back in to your Nectar instance, list running sessions/screens:

```
byobu-screen -ls
```

If it says (Detached) next to the `workshop` session in the list, reattach to `workshop` by:

```
byobu-screen -r workshop
```

If it says (Attached) next to the `workshop` session in the list, you can access `workshop` which is already attached by:

```
byobu-screen -r -d workshop
```

Some other useful `byobu-screen` commands:

- To detach from `workshop`, type `ctrl-a ctrl-d` while inside the `workshop` session.
  (You will need to configure Byobu's ctrl-a behaviour if it hasn't already been configured (text will appear on the screen telling you this). Follow the information on the screen and select `1` for Screen mode).

- To terminate `workshop`, type `ctrl-d` while inside the `workshop` session.

Required Data

- No additional data needs to be downloaded for this workshop - it is all located on the Nectar Instance. FASTQs are located in the directory `raw_data` and a metadata (`metadata.tsv`) file has also been provided.

- If you wish to analyse the data independently at a later stage, it can be downloaded from here. This zipped folder contains both the FASTQs and associated metadata file.

- If you are running this tutorial independently, you can also access the classifier that has been trained specifically for this data from here.

Symbolic links to workshop data

Data for this workshop is stored in a central location (`/mnt/shared_data/`) on the Nectar file system that we will be using. We will use symbolic links (`ln -s`) to point to it. Symbolic links (or symlinks) are just "virtual" files or folders (they only take up a very little space) that point to a physical file or folder located elsewhere in the file system. Sequencing data can be large, and rather than unnecessarily having multiple copies of the data which can quickly take up a lot of space, we will simply point to the files needed in the `shared_data` folder.

```
cd
ln -s /mnt/shared_data/raw_data raw_data
ln -s /mnt/shared_data/metadata.tsv metadata.tsv
ln -s /mnt/shared_data/silva_138_16s_v5v6_classifier_2021-4.qza silva_138_16s_v5v6_classifier_2021-4.qza
```

Slides and workshop instructions

Click here for slides presented during this workshop.

Click here for a printer friendly PDF version of this workshop.

Author Information

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Created/Reviewed: August 2021

Background

What is the influence of genotype (intrinsic) and environment (extrinsic) on anemone-associated bacterial communities?

The Players

- *Exaiptasia diaphana* - a shallow-water, marine anemone that is often used in research as a model organism for corals. In this experiment, two genotypes (AIMS1 and AIMS4) of *E. diaphana* were grown in each of two different environments:

    i. sterile seawater ***OR***

ii. unfiltered control seawater

- The anemone-associated bacterial communities or *microbiome* - these bacteria live on, or within *E. diaphana*, and likely consist of a combination of commensals, transients, and long-term stable members, and combined with their host, form a mutually beneficial, stable symbiosis.

The Study

The anemone microbiome contributes to the overall health of this complex system and can evolve in tandem with the anemone host. In this data set we are looking at the impact of intrinsic and extrinsic factors on anemone microbiome composition. After three weeks in either sterile or control seawater (environment), anemones were homogenized and DNA was extracted. There are *23* samples in this data set - *5* from each anemone treatment combination (2 genotypes x 2 environments) and *3* DNA extraction blanks as controls. *This data is a subset from a larger experiment*.

Dungan AM, van Oppen MJH, and Blackall LL (2021) Short-Term Exposure to Sterile Seawater Reduces Bacterial Community Diversity in the Sea Anemone, *Exaiptasia diaphana*. *Front. Mar. Sci.* 7:599314. doi:10.3389/fmars.2020.599314 [Full Text].

QIIME 2 Analysis platform

Quantitative Insights Into Microbial Ecology 2 (QIIME 2™) is a next-generation microbiome bioinformatics platform that is extensible, free, open source, and community developed. It allows researchers to:

- Automatically track analyses with decentralised data provenance
- Interactively explore data with beautiful visualisations
- Easily share results without QIIME 2 installed
- Plugin-based system — researchers can add in tools as they wish

!!! attention
The version used in this workshop is qiime2-2021.4. Other versions of QIIME2 may result in minor differences in results.

Viewing QIIME2 visualisations

As this workshop is being run on a remote Nectar Instance, you will need to download the visual files (*.qzv) to your local computer and view them in QIIME 2 View (q2view).

!!! attention
We will be doing this step multiple times throughout this workshop to view visualisation files as they are generated.

Mac Users

The syntax to do this depends on whether you are running the copying command on your local computer, or on the remote computer (Nectar cloud).

1. When running the command from your local computer, the syntax for copying a file *from* Nectar is:

```
scp username@nectar_IP_address:FILENAME /PATH/TO/TARGET/FOLDER/
```

2. Running the command on the remote computer, the syntax for copying a file *to* your local computer is:
`bash scp FILENAME username@your_IP_address:/PATH/TO/TARGET/FOLDER/`

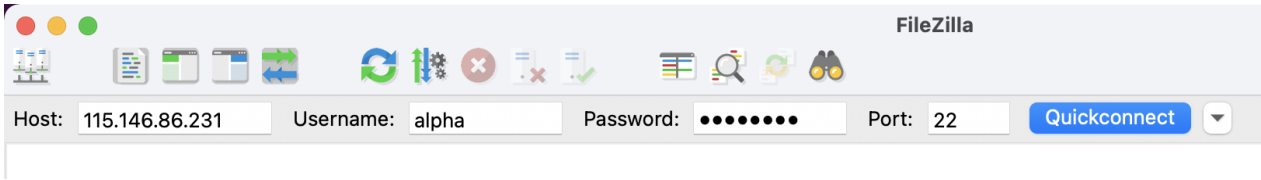
Less experienced Unix users may want to use FileZilla. See section below for more details.

Windows Users

Using WinSCP or FileZilla:

Host: The IP address of the Nectar instance
Username: alpha | beta | gamma | delta | epsilon | zeta
Port: 22

??? example "Filezilla Example"



Alternatively, ***if you have QIIME2 installed and are running it on your own computer***, you can use `qiime tools view` to view the results from the command line (e.g. `qiime tools view filename.qzv`). `qiime tools view` opens a browser window with your visualization loaded in it. When you are done, you can close the browser window and press `ctrl-c` on the keyboard to terminate the command.

Section 1: Importing, cleaning and quality control of the data

Import data

These samples were sequenced on a single Illumina MiSeq run using v3 (2 × 300 bp) reagents at the Walter and Eliza Hall Institute (WEHI), Melbourne, Australia. Data from WEHI came as paired-end, demultiplexed, unzipped *.fastq files with adapters still attached. Following the QIIME2 importing tutorial, this is the Casava One Eight format. The files have been renamed to satisfy the Casava format as SampleID_FWDXX-REVXX_L001_R[1 or 2]_001.fastq e.g. CTRLA_Fwd04-Rev25_L001_R1_001.fastq.gz. The files were then zipped (.gzip).

Here, the data files (two per sample i.e. forward and reverse reads `R1` and `R2` respectively) will be imported and exported as a single QIIME 2 artefact file. These samples are already demultiplexed (i.e. sequences from each sample have been written to separate files), so a metadata file is not initially required.

!!! note
To check the input syntax for any QIIME2 command, enter the command, followed by `--help` e.g. `qiime tools import --help`

!!! attention
If you haven't already done so, make sure you are running the workshop in byobu-screen and have created the symbolic links to the workshop data.

Start by making a new directory `analysis` to store all the output files from this tutorial. In addition, we will create a subdirectory called `seqs` to store the exported sequences.

```
cd
mkdir -p analysis/seqs
```

Run the command to import the raw data located in the directory `raw_data` and export it to a single QIIME 2 artefact file, `combined.qza`.

```
qiime tools import \
--type 'SampleData[PairedEndSequencesWithQuality]' \
--input-path raw_data \
--input-format CasavaOneEightSingleLanePerSampleDirFmt \
--output-path analysis/seqs/combined.qza
```

Remove primers

!!! important
Remember to ask you sequencing facility if the raw data you get has the primers attached - they may have already been removed.

These sequences still have the primers attached - they need to be removed (using `cutadapt`) before denoising.

```
qiime cutadapt trim-paired \
--i-demultiplexed-sequences analysis/seqs/combined.qza \
--p-front-f AGGATTAGATACCCTGGTA \
--p-front-r CRRCACGAGCTGACGAC \
--p-error-rate 0.20 \
--output-dir analysis/seqs_trimmed \
--verbose
```

!!! attention
The primers specified (784f and 1492r for the bacterial 16S rRNA gene) correspond to *this* specific experiment - they will likely not work for your own data analyses.

!!! attention
The error rate parameter, `#!python --p-error-rate`, will likely need to be adjusted for your own sample data to get 100% (or close to it) of reads trimmed.

Create and interpret sequence quality data

Create a viewable summary file so the data quality can be checked. Viewing the quality plots generated here helps determine trim settings.

!!! info "**Things to look for:**"
1. Where does the median quality drop below 30?
2. Do any of the samples have only a few sequences e.g. <1000? If so, you may want to omit them from the analysis later on in R.

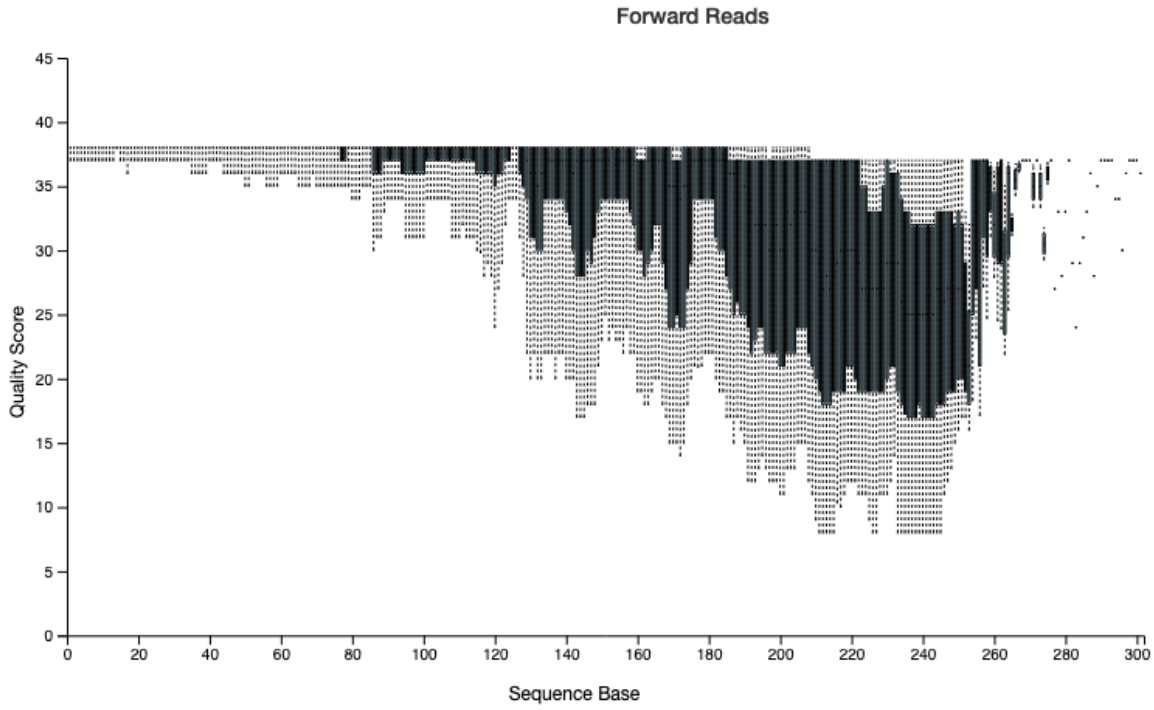
Create a subdirectory in `analysis` called `visualisations` to store all files that we will visualise in one place.

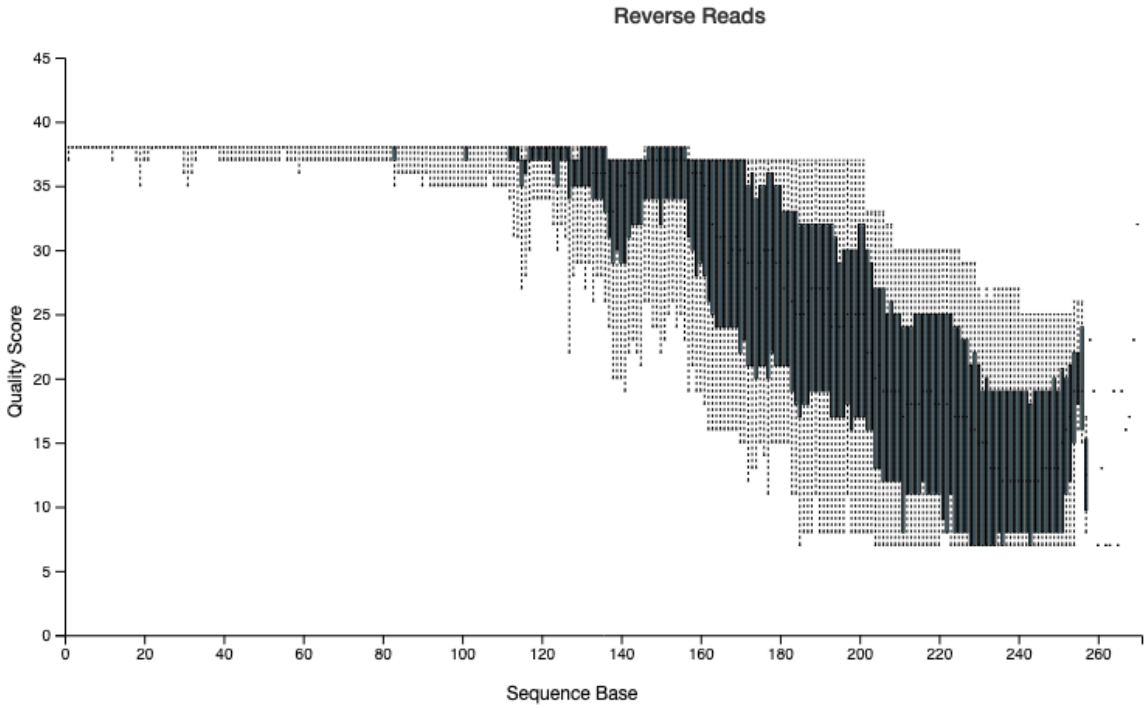
```
mkdir analysis/visualisations
```

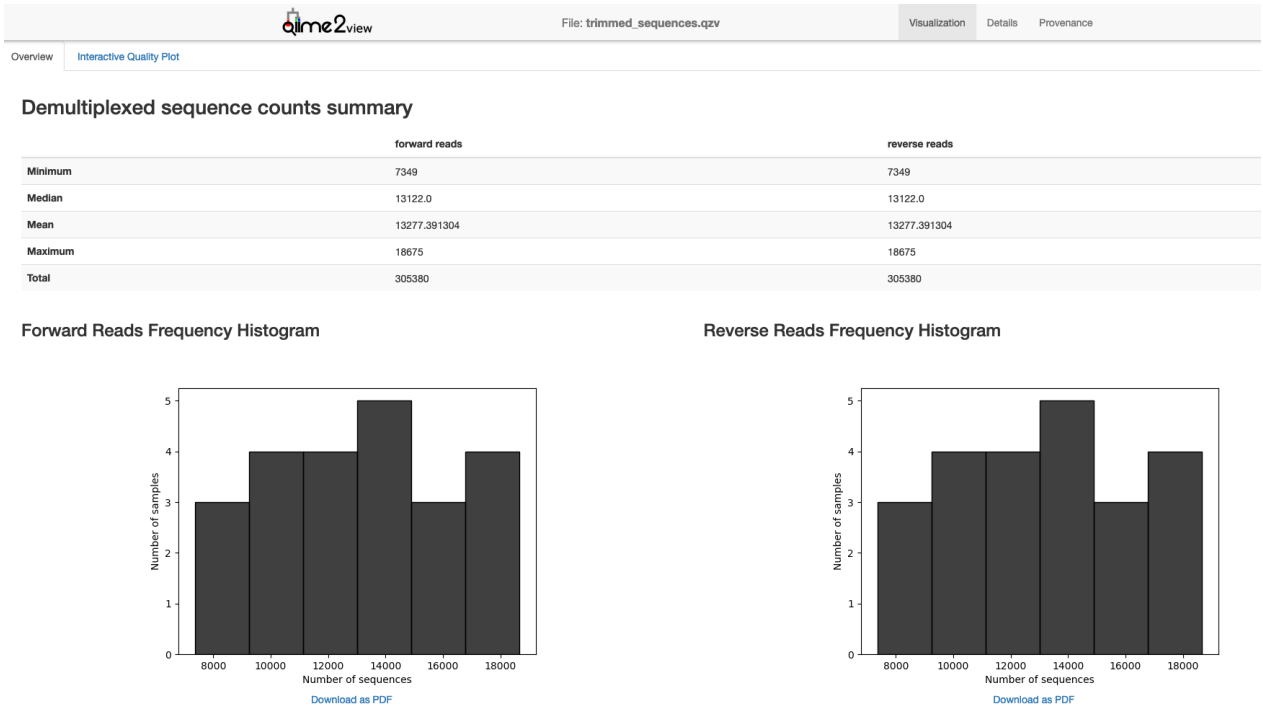
```
qiime demux summarize \
--i-data analysis/seqs_trimmed/trimmed_sequences.qza \
--o-visualization analysis/visualisations/trimmed_sequences.qzv
```

Copy `analysis/visualisations/trimmed_sequences.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisations: Read quality and demux output"







	forward reads	reverse reads
Minimum	7349	7349
Median	13122.0	13122.0
Mean	13277.391304	13277.391304
Maximum	18675	18675
Total	305380	305380

Denoising the data

Trimmed sequences are now quality assessed using the `dada2` plugin within QIIME2. `dada2` denoises data by modelling and correcting Illumina-sequenced amplicon errors, and infers exact amplicon sequence variants (***ASVs***), resolving differences of as little as 1 nucleotide. Its workflow consists of filtering, de-replication, reference-free chimera detection, and paired-end reads merging, resulting in a feature or ***ASV*** table.

!!! note
This step may long time to run (i.e. hours), depending on files sizes and computational power.

```
Remember to adjust `p-trunc-len-f` and `p-trunc-len-r` values according to your own data.
```

!!! question "Question: Based on your assessment of the quality plots from the trimmed_sequences.qzv file generated in the previous step, what values would you select for `p-trunc-len-f` and `p-trunc-len-r` in the command below? *Hint: At what base pair does the median quality drop below 30?*"

```
??? answer
    For version qiime2-2021.4: `p-trunc-len-f 211` and `p-trunc-len-r 172`. Other QIIME2 versions may slightl
```

The specified output directory must not pre-exist.

```
qiime dada2 denoise-paired \
--i-demultiplexed-seqs analysis/seqs_trimmed/trimmed_sequences.qza \
--p-trunc-len-f xx \
--p-trunc-len-r xx \
--p-n-threads 0 \
--output-dir analysis/dada2out \
--verbose
```

Generate summary files

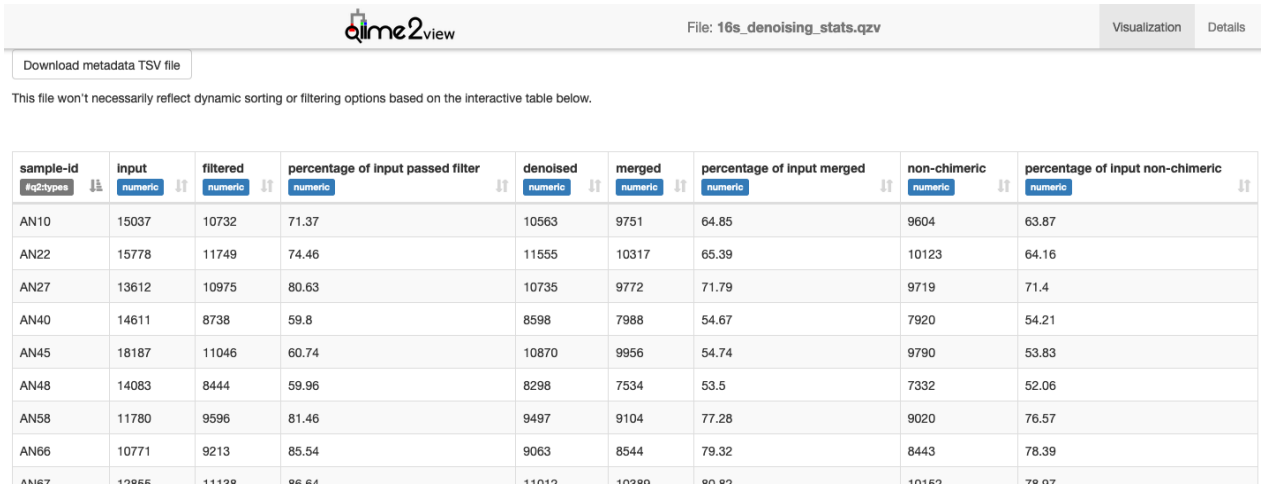
A metadata file is required which provides the key to gaining biological insight from your data. The file metadata.tsv is provided in the home directory of your Nectar instance. This spreadsheet has already been verified using the plugin for Google Sheets, keemei.

!!! info "**Things to look for:**"
1. How many features (*ASVs*) were generated? Are the communities high or low diversity?
2. Do BLAST searches of the representative sequences make sense? Are the features what you would expect e.g. marine or terrestrial?
3. Have a large number (e.g. >50%) of sequences been lost during denoising/filtering? If so, the settings might be too stringent.

```
qiime metadata tabulate \
--m-input-file analysis/dada2out/denoising_stats.qza \
--o-visualization analysis/visualisations/16s_denoising_stats.qzv \
--verbose
```

Copy `analysis/visualisations/16s_denoising_stats.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Denoising Stats"



sample-id #q2:types	input numeric	filtered numeric	percentage of input passed filter numeric	denoised numeric	merged numeric	percentage of input merged numeric	non-chimeric numeric	percentage of input non-chimeric numeric
AN10	15037	10732	71.37	10563	9751	64.85	9604	63.87
AN22	15778	11749	74.46	11555	10317	65.39	10123	64.16
AN27	13612	10975	80.63	10735	9772	71.79	9719	71.4
AN40	14611	8738	59.8	8598	7988	54.67	7920	54.21
AN45	18187	11046	60.74	10870	9956	54.74	9790	53.83
AN48	14083	8444	59.96	8298	7534	53.5	7332	52.06
AN58	11780	9596	81.46	9497	9104	77.28	9020	76.57
AN66	10771	9213	85.54	9063	8544	79.32	8443	78.39
AN67	13855	11138	86.64	11012	10389	80.83	10152	78.97

```
qiime feature-table summarize \
--i-table analysis/dada2out/table.qza \
--m-sample-metadata-file metadata.tsv \
--o-visualization analysis/visualisations/16s_table.qzv \
--verbose
```

Copy `analysis/visualisations/16s_table.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisations: Feature/ASV summary"

qiime2view File: **16s_table.qzv**

Overview | Interactive Sample Detail | Feature Detail

Table summary

Metric	Sample
Number of samples	23
Number of features	554
Total frequency	213,035

Frequency per sample

	Frequency
Minimum frequency	5,583.0
1st quartile	7,626.0
Median frequency	9,020.0
3rd quartile	10,137.5
Maximum frequency	14,021.0
Mean frequency	9,262.391304347826

Frequency per sample detail (csv | html)

qiime2view File: **16s_table.qzv** Visualization Details Provenance

Overview | Interactive Sample Detail | Feature Detail

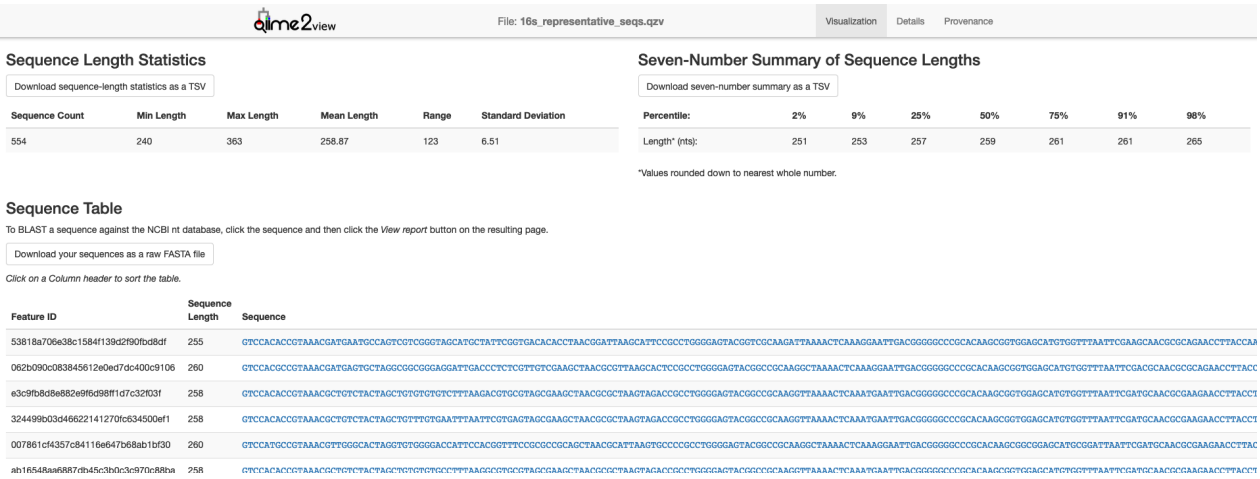
	Frequency	# of Samples Observed In
53818a706e38c1584f139d2f90fbd8df	14,434	18
062b090c083845612e0ed7dc400c9106	10,491	18
e3c9fb8d8e882e9f6d98ff1d7c32f03f	8,771	21
324499b03d46622141270fc634500ef1	7,755	15
007861cf4357c84116e647b68ab1bf30	5,926	5
ab16548aa6887db45c3b0c3c970c88ba	5,419	10
5726619608b84ee3018c815204cdff7a	5,253	14
682e5342211c704576db92e0bb567c8e	5,150	19
e0cc6a95596c1068ec99eb67cea8d93e	4,388	17
036c25714c3a550c732bb3fb065c2637	4,386	12
e82296843639d9044e7f161d5628873e	3,308	7
77cd18f21765bf8edab2287e1498809f	3,120	7

```
qiime feature-table tabulate-seqs \
--i-data analysis/dada2out/representative_sequences.qza \
```

```
  --o-visualization analysis/visualisations/16s_representative_seqs.qzv \
  --verbose
```

Copy `analysis/visualisations/16s_representative_seqs.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Representative Sequences"



Section 2: Taxonomic Analysis

Assign taxonomy

Here we will classify each identical read or *Amplicon Sequence Variant (ASV)* to the highest resolution based on a database. Common databases for bacteria datasets are Greengenes, SILVA, Ribosomal Database Project, or Genome Taxonomy Database. See Porter and Hajibabaei, 2020 for a review of different classifiers for metabarcoding research. The classifier chosen is dependent upon:

1. Previously published data in a field
2. The target region of interest
3. The number of reference sequences for your organism in the database and how recently that database was updated.

A classifier has already been trained for you for the V5V6 region of the bacterial 16S rRNA gene using the SILVA database. The next step will take a while to run. *The output directory cannot previously exist*.

n_jobs = 1 This runs the script using all available cores

!!! note
The classifier used here is only appropriate for the specific 16S rRNA region that *this* data represents. You will need to train your own classifier for your own data. For more information about training your own classifier, see Section 6: Extra Information.

!!! fail "STOP - Workshop participants only"
Due to time limitations in a workshop setting, please do NOT run the `qiime feature-classifier classify-sklearn` command below. You will need to access a pre-computed `classification.qza` file that this command generates by running the following: `cd; mkdir analysis/taxonomy; cp /mnt/shared_data/pre_computed/classification.qza analysis/taxonomy`. If you have accidentally run the command below, `ctrl-z` will terminate it.

```
qiime feature-classifier classify-sklearn \
--i-classifier silva_138_16s_v5v6_classifier_2021-4.qza \
--i-reads analysis/dada2out/representative_sequences.qza \
--p-n-jobs 1 \
--output-dir analysis/taxonomy \
--verbose
```

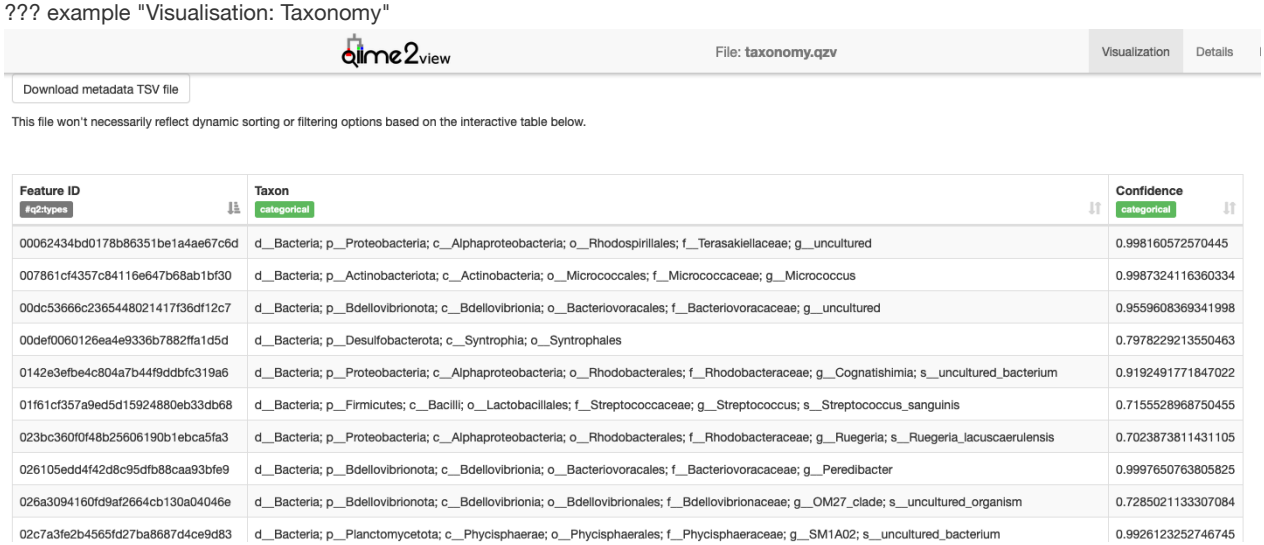
!!! warning "Warning"
This step often runs out of memory on full datasets. Some options are to change the number of cores you are using (adjust `--p-n-jobs`) or add `--p-reads-per-batch 10000` and try again. The QIIME 2 forum has many threads regarding this issue so always check there was well.

Generate a viewable summary file of the taxonomic assignments.

```
qiime metadata tabulate \
--m-input-file analysis/taxonomy/classification.qza \
--o-visualization analysis/visualisations/taxonomy.qzv \
--verbose
```

Copy `analysis/visualisations/taxonomy.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Taxonomy"



Feature ID #q2:types	Taxon categorical	Confidence categorical
00062434bd0178b86351be1a4ae67c6d	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodospirillales; f__Terasakiellaceae; g__uncultured	0.998160572570445
007861cf4357c84116e647b68ab1bf30	d__Bacteria; p__Actinobacteriota; c__Actinobacteria; o__Micrococcales; f__Micrococcaceae; g__Micrococcus	0.9987324116360334
00dc53666c2365448021417f36df12c7	d__Bacteria; p__Bdellovibrionota; c__Bdellovibrionia; o__Bacteriovoracales; f__Bacteriovoracaceae; g__uncultured	0.9559608369341998
00def0060126ea4e9336b7882ffa1d5d	d__Bacteria; p__Desulfobacterota; c__Syntrophia; o__Syntrophales	0.7978229213550463
0142e3efbe4c804a7b44f9ddbfc319a6	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Cognatishimia; s__uncultured_bacterium	0.9192491771847022
01f61cf357a9ed5d15924880eb33db68	d__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Streptococcaceae; g__Streptococcus; s__Streptococcus_sanguinis	0.7155528968750455
023bc360f0f48b25606190b1ebca5fa3	d__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Ruegeria; s__Ruegeria_lacuscaerulensis	0.7023873811431105
026105edd4f42d8c95dfb88caa93bfe9	d__Bacteria; p__Bdellovibrionota; c__Bdellovibrionia; o__Bacteriovoracales; f__Bacteriovoracaceae; g__Peredibacter	0.9997650763805825
026a3094160fd9af2664cb130a04046e	d__Bacteria; p__Bdellovibrionota; c__Bdellovibrionia; o__Bdellovibrionales; f__Bdellovibrionaceae; g__OM27_clade; s__uncultured_organism	0.7285021133307084
02c7a3fe2b4565fd27ba8687d4ce9d83	d__Bacteria; p__Planctomycetota; c__Phycisphaerae; o__Phycisphaerales; f__Phycisphaeraceae; g__SM1A02; s__uncultured_bacterium	0.9926123252746745

Filtering

Filter out reads classified as mitochondria and chloroplast. Unassigned ASVs are retained. Generate a viewable summary file of the new table to see the effect of filtering.

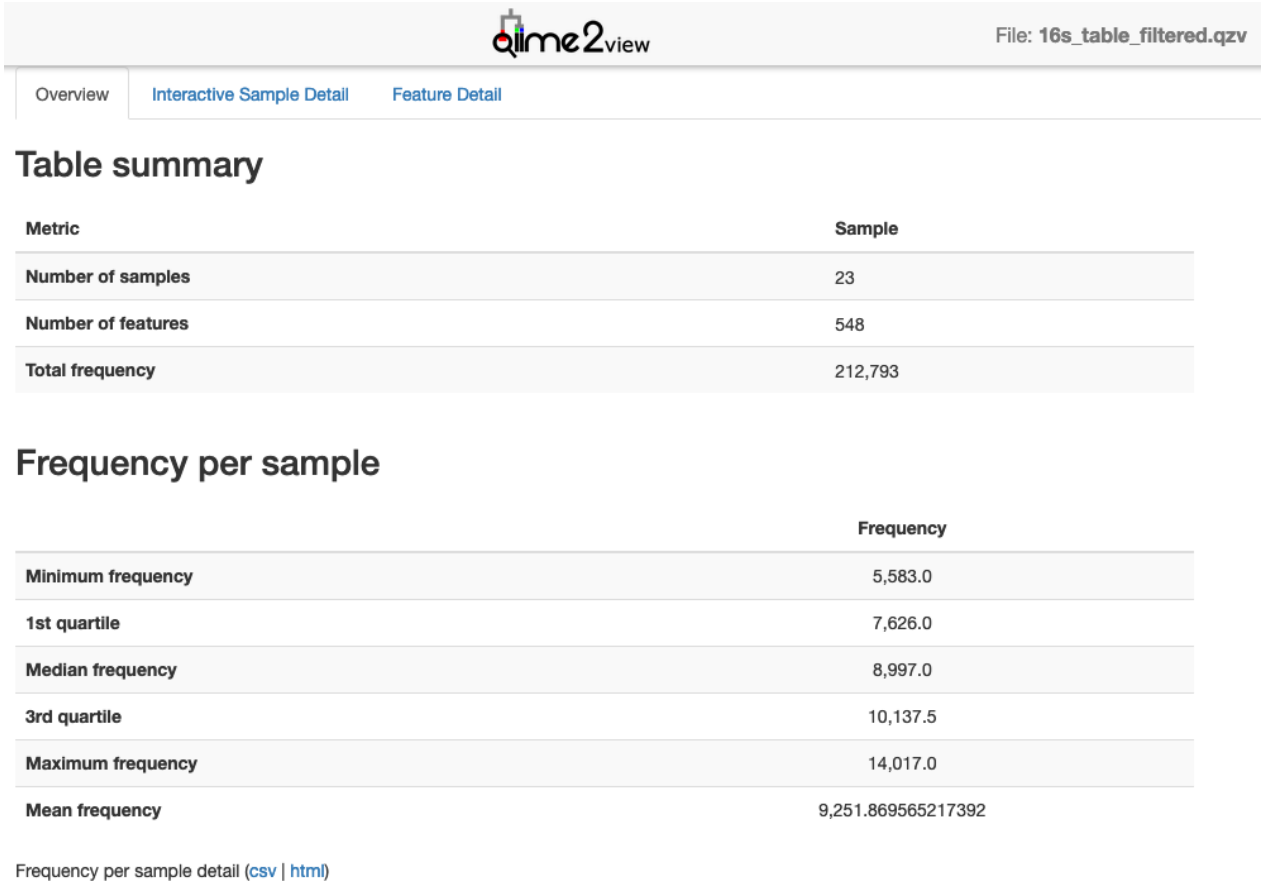
According to QIIME developer Nicholas Bokulich, low abundance filtering (i.e. removing ASVs containing very few sequences) is not necessary under the ASV model.

```
qiime taxa filter-table \
--i-table analysis/dada2out/table.qza \
--i-taxonomy analysis/taxonomy/classification.qza  \
--p-exclude Mitochondria,Chloroplast \
--o-filtered-table analysis/taxonomy/16s_table_filtered.qza \
--verbose
```

```
qiime feature-table summarize \
--i-table analysis/taxonomy/16s_table_filtered.qza \
--m-sample-metadata-file metadata.tsv \
--o-visualization analysis/visualisations/16s_table_filtered.qzv \
--verbose
```

Copy `analysis/visualisations/16s_table_filtered.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: 16s_table_filtered"



qiime2view File: **16s_table_filtered.qzv**

Overview | Interactive Sample Detail | Feature Detail

Table summary

Metric	Sample
Number of samples	23
Number of features	548
Total frequency	212,793

Frequency per sample

	Frequency
Minimum frequency	5,583.0
1st quartile	7,626.0
Median frequency	8,997.0
3rd quartile	10,137.5
Maximum frequency	14,017.0
Mean frequency	9,251.869565217392

Frequency per sample detail (csv | html)

Section 3: Build a phylogenetic tree

The next step does the following:

1. Perform an alignment on the representative sequences.
2. Mask sites in the alignment that are not phylogenetically informative.
3. Generate a phylogenetic tree.
4. Apply mid-point rooting to the tree.

A phylogenetic tree is necessary for any analyses that incorporates information on the relative relatedness of community members, by incorporating phylogenetic distances between observed organisms in the computation. This would include any beta-diversity analyses and visualisations from a weighted or unweighted Unifrac distance matrix.

```
mkdir analysis/tree
```

Use one thread only (which is the default action) so that identical results can be produced if rerun.

```
qiime phylogeny align-to-tree-mafft-fasttree \
--i-sequences analysis/dada2out/representative_sequences.qza \
--o-alignment analysis/tree/aligned_16s_representative_seqs.qza \
--o-masked-alignment analysis/tree/masked_aligned_16s_representative_seqs.qza \
--o-tree analysis/tree/16s_unrooted_tree.qza \
--o-rooted-tree analysis/tree/16s_rooted_tree.qza \
```

```
  --p-n-threads 1 \
  --verbose
```

Section 4: Basic visualisations and statistics

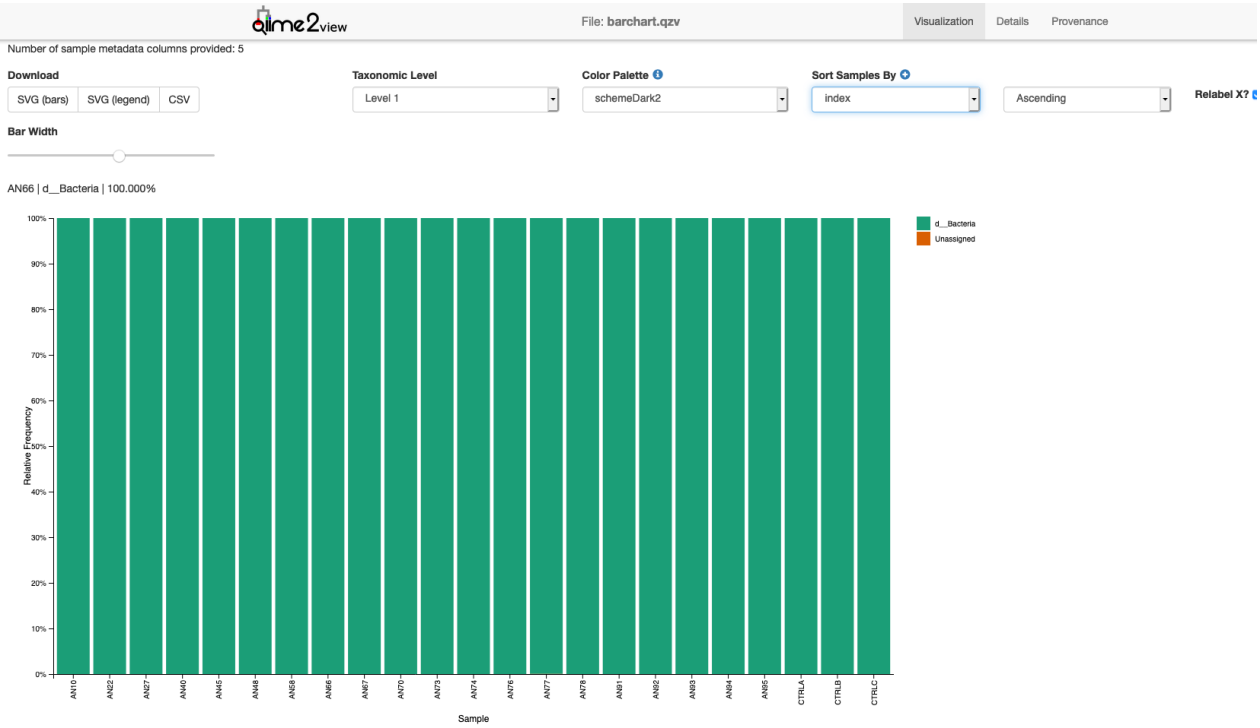
ASV relative abundance bar charts

Create bar charts to compare the relative abundance of ASVs across samples.

```
qiime taxa barplot \
--i-table analysis/taxonomy/16s_table_filtered.qza \
--i-taxonomy analysis/taxonomy/classification.qza \
--m-metadata-file metadata.tsv \
--o-visualization analysis/visualisations/barchart.qzv \
--verbose
```

Copy `analysis/visualisations/barchart.qzv` to your local computer and view in QIIME 2 View (q2view). Try selecting different taxonomic levels and metadata-based sample sorting.

??? example "Visualisations: Taxonomy Barplots"




```
![barplot2](./media/barplot_level3.png)

![barplot3](./media/barplot_level5.png)
```

Rarefaction curves

Generate rarefaction curves to determine whether the samples have been sequenced deeply enough to capture all the community members. The max depth setting will depend on the number of sequences in your samples.

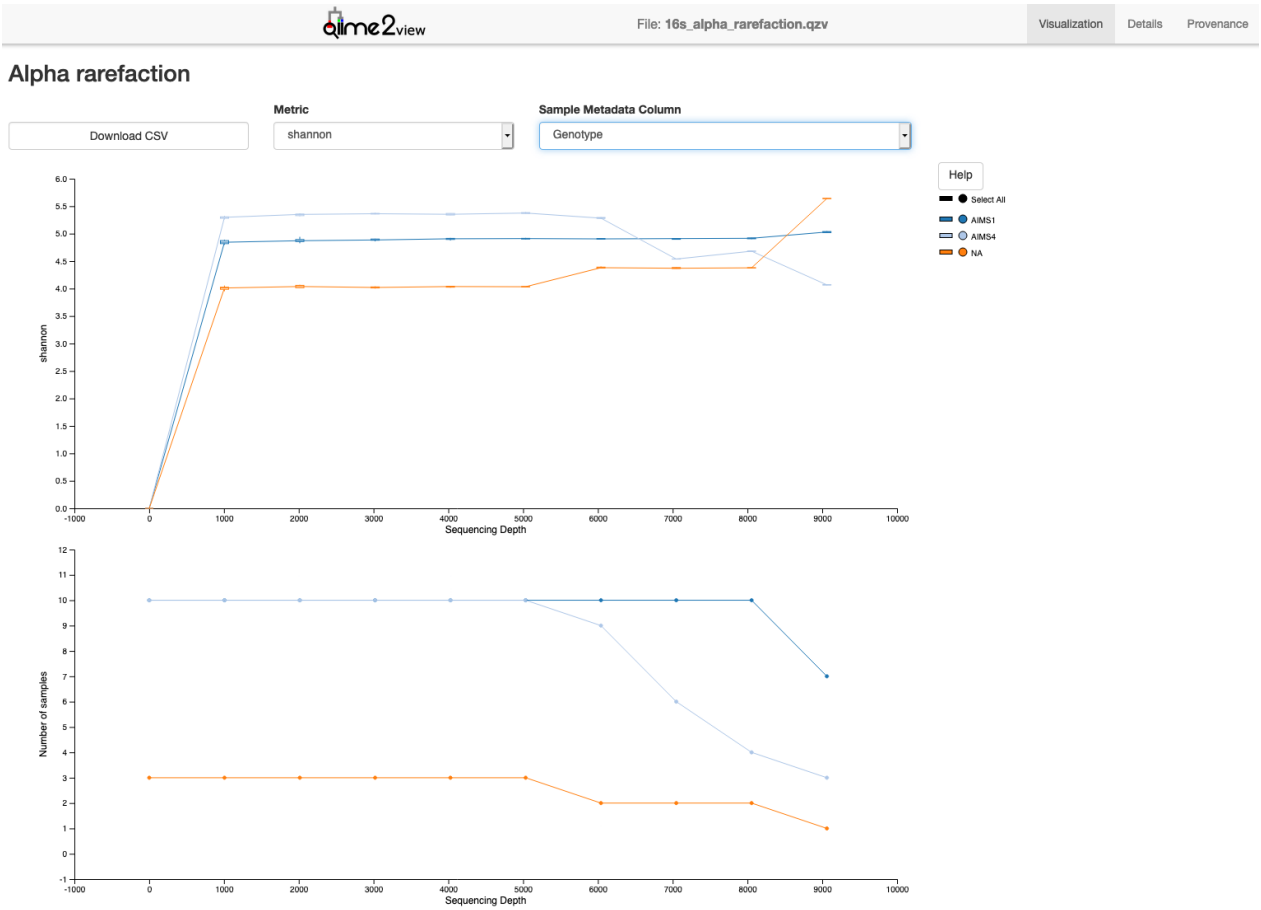
!!! info "**Things to look for:**"
1. Do the curves for each sample plateau? If they don't, the samples haven't been sequenced deeply enough to capture the full diversity of the bacterial communities, which is shown on the y-axis.
2. At what sequencing depth (x-axis) do your curves plateau? This value will be important for downstream analyses, particularly for alpha diversity analyses.

!!! note
The value that you provide for --p-max-depth should be determined by reviewing the "Frequency per sample" information presented in the 16s_table_filtered.qzv file that was created above. In general, choosing a value that is somewhere around the median frequency seems to work well, but you may want to increase that value if the lines in the resulting rarefaction plot don't appear to be leveling out, or decrease that value if you seem to be losing many of your samples due to low total frequencies closer to the minimum sampling depth than the maximum sampling depth.

```
qiime diversity alpha-rarefaction \
--i-table analysis/taxonomy/16s_table_filtered.qza \
--i-phylogeny analysis/tree/16s_rooted_tree.qza \
--p-max-depth 9062 \
--m-metadata-file metadata.tsv \
--o-visualization analysis/visualisations/16s_alpha_rarefaction.qzv \
--verbose
```

Copy `analysis/visualisations/16s_alpha_rarefaction.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Rarefaction"



Alpha and beta diversity analysis

The following is taken directly from the Moving Pictures tutorial and adapted for this data set. QIIME 2's diversity analyses are available through the `q2-diversity` plugin, which supports computing alpha- and beta- diversity metrics, applying related

statistical tests, and generating interactive visualisations. We'll first apply the core-metrics-phylogenetic method, which rarefies a FeatureTable[Frequency] to a user-specified depth, computes several alpha- and beta- diversity metrics, and generates principle coordinates analysis (PCoA) plots using Emperor for each of the beta diversity metrics.

The metrics computed by default are:

- Alpha diversity (operate on a single sample (i.e. within sample diversity)).
  - Shannon's diversity index (a quantitative measure of community richness)
  - Observed OTUs (a qualitative measure of community richness)
  - Faith's Phylogenetic Diversity (a qualitative measure of community richness that incorporates phylogenetic relationships between the features)
  - Evenness (or Pielou's Evenness; a measure of community evenness)
- Beta diversity (operate on a pair of samples (i.e. between sample diversity)).
  - Jaccard distance (a qualitative measure of community dissimilarity)
  - Bray-Curtis distance (a quantitative measure of community dissimilarity)
  - unweighted UniFrac distance (a qualitative measure of community dissimilarity that incorporates phylogenetic relationships between the features)
  - weighted UniFrac distance (a quantitative measure of community dissimilarity that incorporates phylogenetic relationships between the features)

An important parameter that needs to be provided to this script is `--p-sampling-depth`, which is the even sampling (i.e. rarefaction) depth that was determined above. As most diversity metrics are sensitive to different sampling depths across different samples, this script will randomly subsample the counts from each sample to the value provided for this parameter. For example, if `--p-sampling-depth 500` is provided, this step will subsample the counts in each sample without replacement, so that each sample in the resulting table has a total count of 500. If the total count for any sample(s) are smaller than this value, those samples will be excluded from the diversity analysis. Choosing this value is tricky. We recommend making your choice by reviewing the information presented in the 16s_table_filtered.qzv file that was created above. Choose a value that is as high as possible (so more sequences per sample are retained), while excluding as few samples as possible.

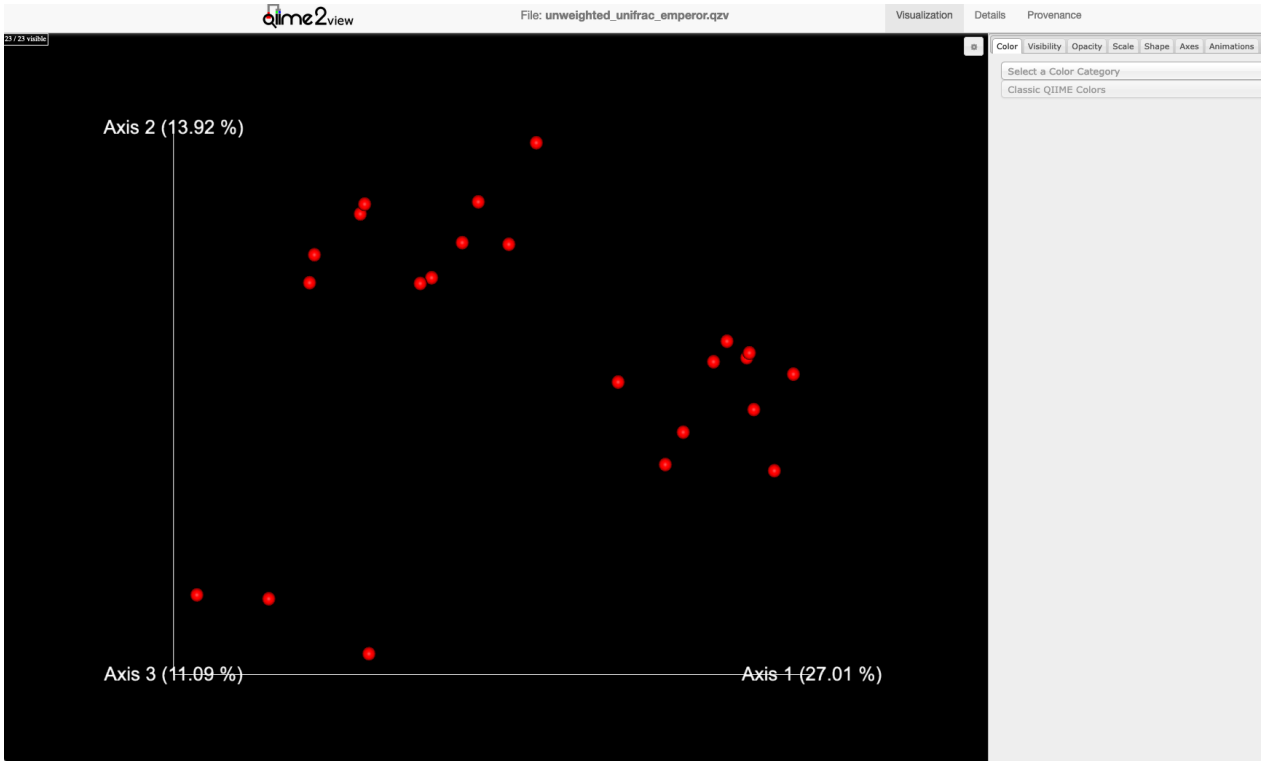
```
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny analysis/tree/16s_rooted_tree.qza \
  --i-table analysis/taxonomy/16s_table_filtered.qza \
  --p-sampling-depth 5583 \
  --m-metadata-file metadata.tsv \
  --output-dir analysis/diversity_metrics
```

Copy the `.qzv` files created from the above command into the `visualisations` subdirectory.

```
cp analysis/diversity_metrics/*.qzv analysis/visualisations
```

To view the differences between sample composition using unweighted UniFrac in ordination space, copy `analysis/visualisations/unweighted_unifrac_emperor.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisations: Unweighted UniFrac Emperor Ordination"



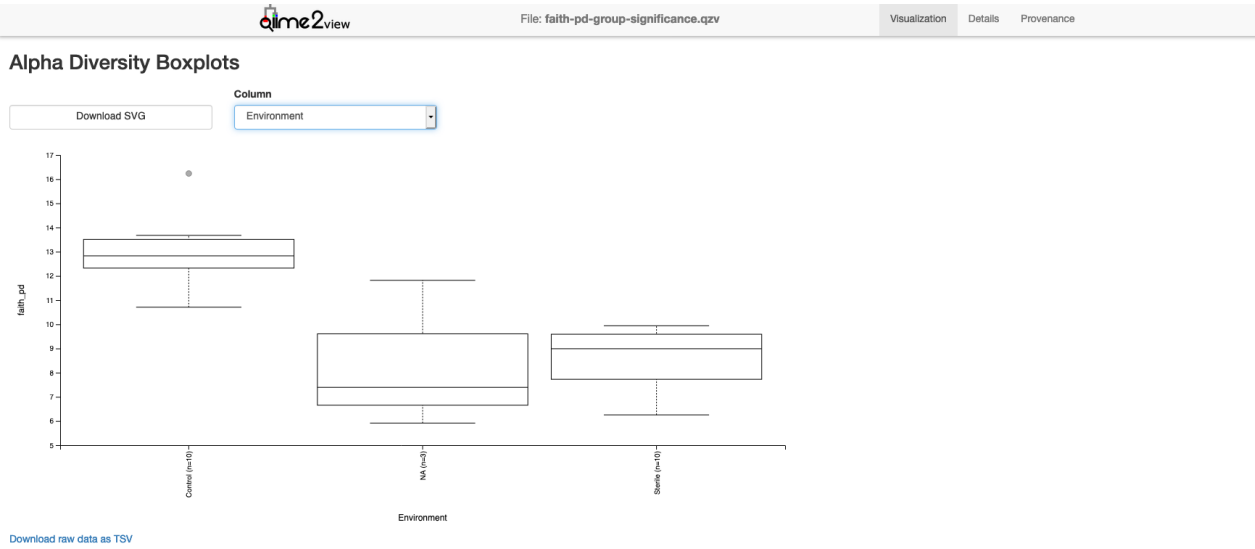

```
On q2view, select the "Colour" tab and the heading "Environment" in the dropdown menu and then by "Genotype"

![unweighted_unifrac_emperor2](./media/unweighted_unifrac_emperor2.png)
```

Next, we'll test for associations between categorical metadata columns and alpha diversity data. We'll do that here for the Faith Phylogenetic Diversity (a measure of community richness) and evenness metrics.

```
qiime diversity alpha-group-significance \
  --i-alpha-diversity analysis/diversity_metrics/faith_pd_vector.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization analysis/visualisations/faith-pd-group-significance.qzv
```

Copy `analysis/visualisations/faith-pd-group-significance.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Faith Phylogenetic Diversity output"



Kruskal-Wallis (all groups)

	Result
H	15.800724637681157
p-value	0.0003706092374210874

Kruskal-Wallis (pairwise)

Download CSV

		H	p-value	q-value
Group 1	**Group 2**			
Control (n=10)	**NA (n=3)**	5.600000	0.017960	0.026941
	Sterile (n=10)	14.285714	0.000157	0.000471
NA (n=3)	**Sterile (n=10)**	0.257143	0.612090	0.612090

```
qiime diversity alpha-group-significance \
  --i-alpha-diversity analysis/diversity_metrics/evenness_vector.qza \
  --m-metadata-file metadata.tsv \
  --o-visualization analysis/visualisations/evenness-group-significance.qzv
```

Copy `analysis/visualisations/evenness-group-significance.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Evenness output"

Finally, we'll analyse sample composition in the context of categorical metadata using a permutational multivariate analysis of variance (PERMANOVA, first described in Anderson (2001)) test using the beta-group-significance command. The following commands will test whether distances between samples within a group, such as samples from the same genotype, are more similar to each other then they are to samples from the other groups. If you call this command with the `--p-pairwise` parameter, as we'll do here, it will also perform pairwise tests that will allow you to determine which specific pairs of groups (e.g., AIMS1 and AIMS4) differ from one another, if any. This command can be slow to run, especially when passing `--p-pairwise` , since it is based on permutation tests. So, unlike the previous commands, we'll run beta-group-significance on specific columns of metadata that we're interested in exploring, rather than all metadata columns to which it is applicable. Here we'll apply this to our unweighted UniFrac distances, using two sample metadata columns, as follows.

```
qiime diversity beta-group-significance \
  --i-distance-matrix analysis/diversity_metrics/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Genotype \
  --o-visualization analysis/visualisations/unweighted-unifrac-genotype-significance.qzv \
  --p-pairwise
```

Copy `analysis/visualisations/unweighted-unifrac-genotype-significance.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Genotype significance output"

File: **unweighted-unifrac-genotype-significance.qzv**

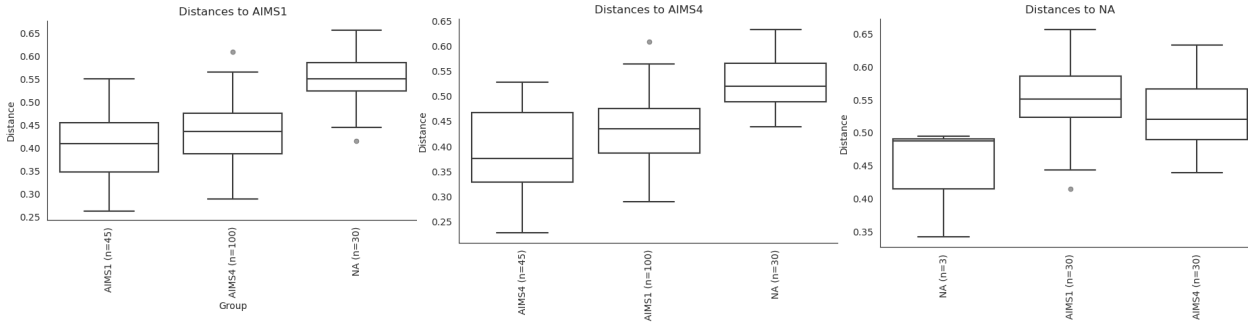
Visualization | Details | Provenance

Overview

	PERMANOVA results
method name	PERMANOVA
test statistic name	pseudo-F
sample size	23
number of groups	3
test statistic	3.506701
p-value	0.001
number of permutations	999

Group significance plots

Download raw data as TSV



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Pairwise permanova results

Download CSV

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
AIMS1	**AIMS4**	20	999	2.649154	0.010	0.010
	NA	13	999	4.257935	0.002	0.006
AIMS4	**NA**	13	999	4.011886	0.006	0.009

```
qiime diversity beta-group-significance \
  --i-distance-matrix analysis/diversity_metrics/unweighted_unifrac_distance_matrix.qza \
  --m-metadata-file metadata.tsv \
  --m-metadata-column Environment \
  --o-visualization analysis/visualisations/unweighted-unifrac-environment-significance.qzv \
  --p-pairwise
```

Copy `analysis/visualisations/unweighted-unifrac-environment-significance.qzv` to your local computer and view in QIIME 2 View (q2view).

??? example "Visualisation: Environmental significance output"

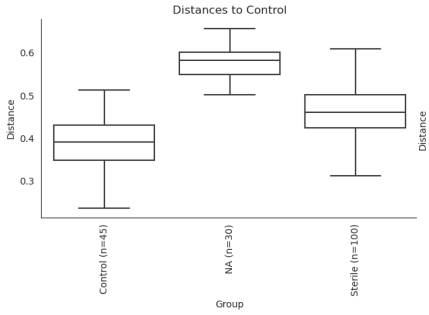
qiime2 view File: **unweighted-unifrac-environment-significance.qzv** Visualization Details Provenance

Overview

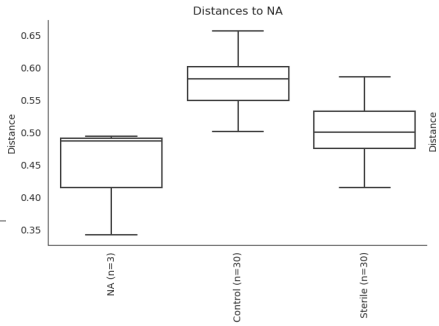
	PERMANOVA results
method name	PERMANOVA
test statistic name	pseudo-F
sample size	23
number of groups	3
test statistic	5.896316
p-value	0.001
number of permutations	999

Group significance plots

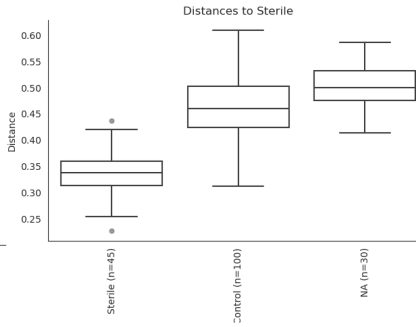
Download raw data as TSV



Download as PDF



Download as PDF



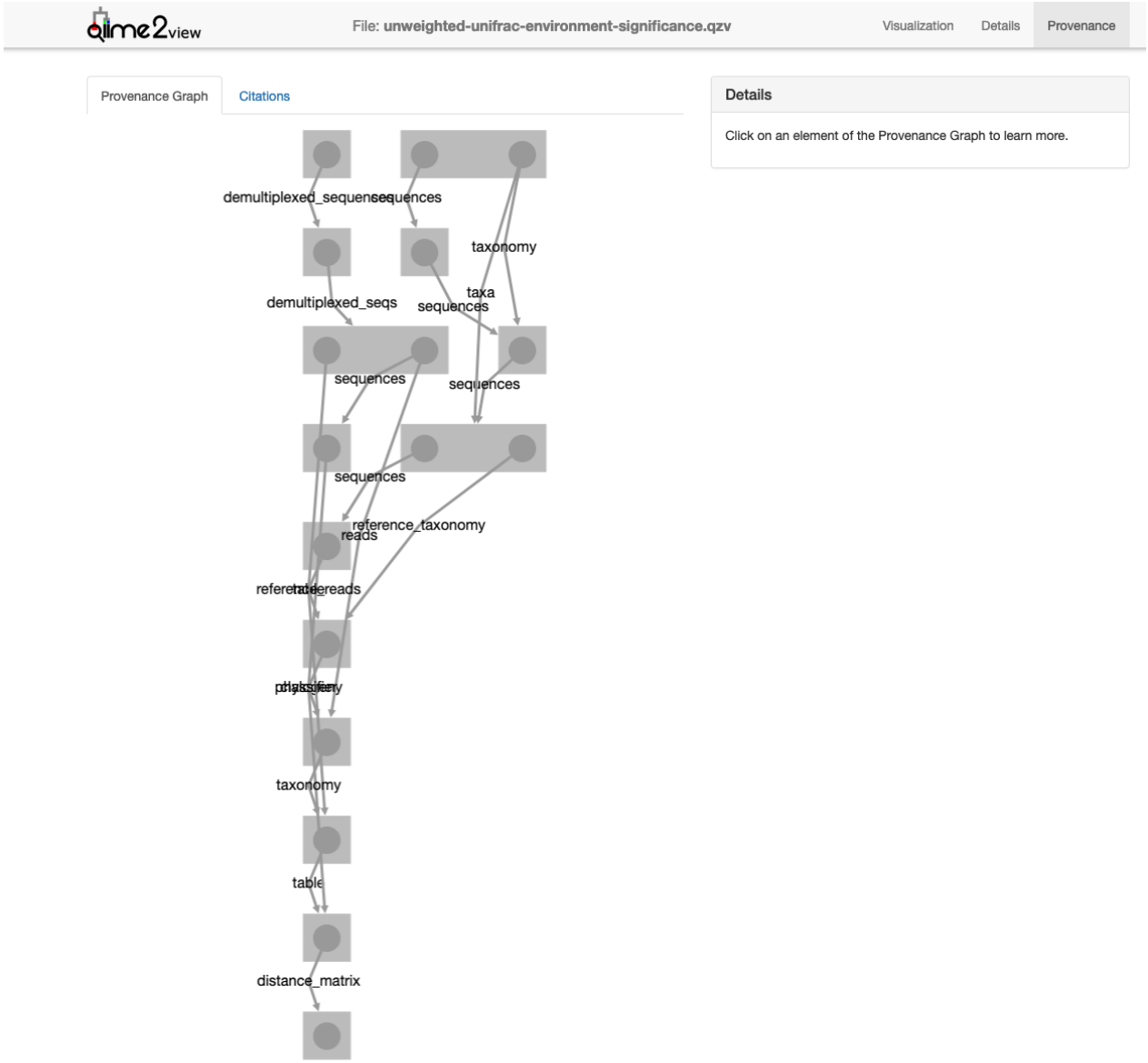
Download as PDF

Pairwise permanova results

Download CSV

Group 1	Group 2	Sample size	Permutations	pseudo-F	p-value	q-value
Control	**NA**	13	999	5.575155	0.002	0.003
	Sterile	20	999	6.895129	0.001	0.003
NA	**Sterile**	13	999	4.676336	0.009	0.009

??? example "Provenance"



Section 5: Exporting data for further analysis in R

You need to export your ASV table, taxonomy table, and tree file for analyses in R. Many file formats can be accepted.

Export unrooted tree as `.nwk` format as required for the R package `phyloseq`.

```
qiime tools export \
  --input-path analysis/tree/16s_unrooted_tree.qza \
  --output-path analysis/export
```

Create a BIOM table with taxonomy annotations. A FeatureTable[Frequency] artefact will be exported as a BIOM v2.1.0 formatted file.

```
qiime tools export \
  --input-path analysis/taxonomy/16s_table_filtered.qza \
```

```
  --output-path analysis/export
```

Then export BIOM to TSV

```
biom convert \
-i analysis/export/feature-table.biom \
-o analysis/export/feature-table.tsv \
--to-tsv
```

Export Taxonomy as TSV

```
qiime tools export \
--input-path analysis/taxonomy/classification.qza \
--output-path analysis/export
```

Delete the header lines of the .tsv files

```
sed '1d' analysis/export/taxonomy.tsv > analysis/export/taxonomy_noHeader.tsv
sed '1d' analysis/export/feature-table.tsv > analysis/export/feature-table_noHeader.tsv
```

Some packages require your data to be in a consistent order, i.e. the order of your ASVs in the taxonomy table rows to be the same order of ASVs in the columns of your ASV table. It's recommended to clean up your taxonomy file. You can have blank spots where the level of classification was not completely resolved.

Section 6: Extra Information

Train SILVA v138 classifier for 16S/18S rRNA gene marker sequences.

The newest version of the SILVA database (v138) can be trained to classify marker gene sequences originating from the 16S/18S rRNA gene. Reference files `silva-138-99-seqs.qza` and `silva-138-99-tax.qza` were downloaded from SILVA and imported to get the artefact files. You can download both these files from here.

Reads for the region of interest are first extracted. **You will need to input your forward and reverse primer sequences**. See QIIME2 documentation for more information.

```
qiime feature-classifier extract-reads \
--i-sequences silva-138-99-seqs.qza \
--p-f-primer FORWARD_PRIMER_SEQUENCE \
--p-r-primer REVERSE_PRIMER_SEQUENCE \
--o-reads silva_138_marker_gene.qza \
--verbose
```

The classifier is then trained using a naive Bayes algorithm. See QIIME2 documentation for more information.

```
qiime feature-classifier fit-classifier-naive-bayes \
--i-reference-reads silva_138_marker_gene.qza \
--i-reference-taxonomy silva-138-99-tax.qza \
--o-classifier silva_138_marker_gene_classifier.qza \
--verbose
```